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Original Article

Serum biomarkers for acute hepatotoxicity of *Echis pyramidum* snake venom in rats

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Abstract: *Echis pyramidum* is a venomous viper responsible for most cases of envenomation in Arabian Peninsula. We determined the acute phase (3-6 h) changes in serum markers of liver function including alanine aminotransferase (ALT), alkaline phosphatase (ALP), gamma glutamyl transferase (GGT) and bilirubin in adult male Sprague Dawley rats injected with *Echis pyramidum* venom (EPV) in the doses of 0.00 (control), 0.25, 0.50 and 1.00 mg/kg bodyweight. We also analyzed markers of oxidative stress including superoxide dismutase (SOD), catalase (CAT), total thiols (T-SH) and thiobarbituric acids reactive substances (TBARS) in liver. The results showed significant and dose- and time-dependent increases in serum ALT, ALP and GGT activities after a single injection of EPV. Serum bilirubin was significantly increased by medium and high doses of EVP after 3 h post-injection and then decreased at 6 h. The low dose of EPV neither affected the activity of SOD nor altered the levels of liver T-SH and TBARS, however, it significantly decreased the activity of CAT at 6 h post-injection of EPV. The medium dose of EPV significantly reduced liver SOD activity after 6 h whereas the high dose significantly reduced the SOD activity at 3 h and 6 h post-dosing. Both medium and high doses of EPV caused significant as well as dose- and time-dependent reductions in liver CAT activities. The high dose significantly reduced T-SH and increased TBARS in rat liver. Further studies are warranted to test the pharmacological potential of early phase antioxidant therapy for neutralizing the toxic effects of EPV.

Keywords: *Echis pyramidum*, snake venom, liver function, antioxidant enzymes, lipid peroxidation, rat

Introduction

Three species of Echis genus including *Echis pyramidum*, *Echis colorotus* and *Echis carinatus sochureki* are distributed throughout the Arabian Peninsula and have been linked with most cases of envenomation in this region [1, 2]. The snakes belonging to Echis genus are poisonous vipers whose lethality is mainly attributed to the highly active enzymatic component, phospholipase A_2 (PLA_2) that hydrolyzes cellular phospholipids thereby releasing arachidonic acid. Oxidative metabolism of arachidonic acid results in the formation of potentially toxic reactive oxygen species (ROS) including superoxide and hydroxyl radicals [3, 4]. Excessive generation of ROS during arachidonic acid metabolism produces lipid peroxides leading to cellular injury [5, 6]. PLA_2 from snake venom has been implicated in multiple pathologies including hepatotoxicity [7]. It has been shown earlier that *Echis pyramidum* venom (EPV) produces significant oxidative stress in different organs of mice, while the onset of lipid peroxidation was as early as 1 h and persisted for several hours, suggesting an important role of oxidative stress in the cytotoxicity of EPV [8]. Although the hepatotoxicity of EPV has been reported in rats [9], the effect of EPV on serum biomarkers of liver function was never studied. In this study, we investigated the effects of EPV on serum levels of bilirubin and activities of alanine aminotransferase (ALT), alkaline phosphatase (ALP) and gamma glutamyl transferase (GGT). We also determined the activities of antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT) as well as non-enzymatic biomarkers of oxidative stress in livers of these animals.

Material and methods

Venom collection

Echis pyramidum snakes were collected from the wild by professional hunters and kept in the serpentarium. EPV was collected from these snakes, filtered, lyophilized and stored at 4°C. A stock solution of EPV (10 mg/ml) was prepared in sterile saline and used for this study.

Animals and dosing

Adult male Sprague Dawley rats weighing 200 ± 20 g were maintained at 23 ± 1°C with 12 h light-dark cycles and free access to standard laboratory food and tap water. The experimental protocol was approved by our Institutional Research and Ethics Committee. The animals were divided into 7 groups of 6 animals in each group. One of these groups served as control and received intraperitoneal (IP) injection of saline. The remaining 6 groups were sub-divided into time-course study (3 h and 6 h) and treated with EPV in the IP doses of 0.25, 0.50 and 1.00 mg/kg bodyweight, respectively. The rats were sacrificed at 3 h or 6 h post-dosing and specimens of blood and livers were collected. The sera and liver samples were promptly stored at -20°C until analyzed.

Serum biomarkers of liver function

The enzymatic activities of alanine aminotransferase (ALT), alkaline phosphatase (ALP) and gamma glutamyl transferase (GGT) and the levels of bilirubin in sera were determined by commercially available kits (United Diagnostic Industries, Dammam, Saudi Arabia) according to manufacturer's instructions.

Antioxidant enzymes activities in liver

The activity of SOD in liver tissues was measured by the method of Marklund and Marklund [10]. To 0.05 ml of supernatant 2.85 ml of 0.05 mM Tris-buffer, pH 8.2 was added, mixed well and incubated at 25°C for 20 min. The reaction was started by adding 0.1 ml of 8 mM pyrogallol solution. Change in absorbance per minute was immediately recorded for the initial 3 min at 420 nm. A reference set, containing distilled water instead of supernatant solution was also run simultaneously.

The activity of CAT in liver was assayed according to the method of Clairborne [11]. The assay mixture consisted of 1.95 ml of 0.05 M potassium phosphate buffer pH 7.0, 1 ml of 0.019 M hydrogen peroxide and 0.05 ml homogenate in a final volume of 3 ml. The decrease in absorbance at 240 nm was recorded immediately and then after every 30 s for 3 min. Enzymatic activity was calculated using the molar extinction coefficient of H_2O_2 (436 M^{-1} cm^{-1} at 240 nm).

Non-enzymatic markers of oxidative stress in liver

The levels of TBARS, a marker of lipid peroxidation [12-14], were estimated in liver tissues using the method of Nichans and Samuelson [15]. Briefly, 0.1 ml of tissue homogenate (Tris-HCl buffer, pH 7.5) was treated with 2 ml of TBA-TCA-HCl (1:1:1) reagent (0.37% TBA, 0.25 N HCl and 15% TCA), placed in a boiling water bath for 15 min, and then cooled. The absorbance of the clear supernatant was measured against a reference blank at 535 nm.

The levels of T-SH, a marker of antioxidant capacity [16, 17], were determined in liver tissues by the method of Sedlak and Lindsay [18]. To 0.4 ml of 10% tissue homogenate, 2.1 ml of 0.1 M Tris-HCl (pH 8.2), 0.5 ml of 10% SDS and 0.3 ml of 0.1 M EDTA were added. The reaction was incubated in a boiling water bath for 5 min. Then, 0.1 ml DTNB (40 mg/100 ml methanol) was added and the absorbance was recorded at 412 nm after 30 min at room temperature. A calibration curve with different amounts of cysteine (20-160 nmoles) was constructed by the same procedure as described above and used to calculate T-SH levels in samples.

Statistical analysis

The data were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test, using SPSS statistical package. *P* value < 0.05 was considered as statistically significant.

Results

There were significant and dose- and time-dependent increases in serum ALT activity after a single IP injection of EPV (ANOVA F = 20.66, P

Table 1. Effect of EPV (3 h and 6 h post-dosing) on serum biomarkers of liver function

Group	Time	ALT (U/L)	ALP (U/L)	GGT (U/L)	Bilirubin (mg/L)
Control		26.76 ± 2.63	186.6 ± 4.84	3.78 ± 0.25	1.50 ± 0.34
EPV 0.25 mg/kg	3 h	33.28 ± 1.51	214.7 ± 5.39	4.23 ± 0.23	3.54 ± 0.54
	6 h	36.41 ± 2.15**	230.3 ± 5.44*	4.83 ± 0.18*	3.34 ± 0.43
EPV 0.50 mg/kg	3 h	35.11 ± 0.79**	222.7 ± 5.82*	4.58 ± 0.50*	6.94 ± 0.92***
	6 h	48.78 ± 2.64***	245.2 ± 5.36**	5.76 ± 0.29***	3.88 ± 0.72
EPV 1.00 mg/kg	3 h	39.87 ± 2.22***	230.8 ± 4.87*	5.47 ± 0.26***	9.12 ± 1.01***
	6 h	52.75 ± 1.10***	273.4 ± 9.06***	7.04 ± 0.24***	4.82 ± 0.63*

Values are means ± SEM. *P < 0.05, **P < 0.01 and ***P < 0.001 versus control group using Dunnett's test.

Table 2. Effect of EPV (3 h and 6 h post-dosing) on markers of oxidative stress in rat liver

Group	Time	SOD (U/g)	CAT (U/g)	T-SH (μmol/g)	TBARS (nmol/g)
Control		10.92 ± 0.84	91.43 ± 5.53	6.83 ± 0.28	66.78 ± 1.10
EPV 0.25 mg/kg	3 h	10.61 ± 0.13	77.05 ± 2.56	6.48 ± 0.08	76.53 ± 0.83
	6 h	10.43 ± 0.16	73.20 ± 5.75*	6.37 ± 0.21	68.14 ± 2.50
EPV 0.50 mg/kg	3 h	10.52 ± 0.53	74.37 ± 4.09*	6.37 ± 0.20	83.15 ± 1.18
	6 h	9.84 ± 0.18**	62.74 ± 4.27**	6.22 ± 0.24	78.92 ± 1.92
EPV 1.00 mg/kg	3 h	9.89 ± 0.22**	72.51 ± 3.55*	5.67 ± 0.25**	116.6 ± 16.7**
	6 h	9.11 ± 0.29***	57.65 ± 4.89***	5.47 ± 0.29**	127.1 ± 11.4***

Values are means ± SEM. *P < 0.05, **P < 0.01 and ***P < 0.001 versus control group using Dunnett's test.

< 0.001) (**Table 1**). Serum ALP (ANOVA F = 8.14, P < 0.001) and GGT (ANOVA F = 22.56, P < 0.001) activities were also significantly increased in dose- and time-dependent manner (**Table 1**). Serum bilirubin was significantly increased by medium (0.50 mg/kg) and high (1.00 mg/kg) doses of EVP at 3 h post-injection and then decreased at 6 h (ANOVA F = 9.29, P < 0.001) (**Table 1**).

The low dose of EPV neither affected the activity of SOD nor altered the levels of liver T-SH and TBARS, however, it significantly decreased the activity of CAT after 6 h post-injection of EPV (**Table 2**). The medium dose of EPV significantly reduced liver SOD activity after 6 h whereas the high dose significantly reduced the SOD activity at 3 h and 6 h post-dosing (ANOVA F = 11.84, P < 0.001) (**Table 2**). Both medium and high doses of EPV caused significant as well as dose- and time-dependent reductions in liver CAT activities (ANOVA F = 5.737, P < 0.01). The low and medium doses of EPV did not produce any significant change in the levels of liver T-SH and TBARS (**Table 2**). Whereas the high dose of EPV significantly reduced T-SH (ANOVA F = 4.01, P < 0.01) and increased TBARS (ANOVA F = 9.42, P < 0.001) in rat liver (**Table 2**).

Discussion

The results of this study showed that IP injection of EPV caused rapid impairment in the liver function as indicated by significant increases in serum ALT, ALP and GGT activities and bilirubin levels (**Table 1**). The hepatic injury was accompanied with significant and dose-dependent reductions in the activities of antioxidant enzymes, SOD and CAT, in rat liver (**Table 2**). EPV envenomation also caused significant depletion of T-SH and elevation of TBARS in liver (**Table 2**). These findings indicate a state of acute oxidative stress within hours following EPV envenomation. Snake venom-induced lipid peroxidation and tissue injury has been reported in vivo [19] and in vitro [20]. Al Asmari et al [8] have shown significant increase in lipid peroxidation in liver, lung, and heart of mice, after 3 h of EPV injection. In vitro exposure of snake venom has been found to generate intracellular ROS in the endothelial cells leading to apoptosis which was accelerated by buthionine sulfoximine and diethyldithiocarbamate, indicating that intracellular glutathione and superoxide levels play a critical role in venom toxicity [21]. Direct exposure of viper venom to human blood for 2 h significantly increased the stress markers, cytoplasmic, lysosomal and extracellular

matrix-degrading enzymes as well as the pro-inflammatory mediators indicating a state of acute oxidative stress and inflammation [22].

EPV-induced lipid peroxidation in liver could be attributed to increased availability of fatty acids that vehemently mobilized from the degradation of phospholipids in presence of PLA_2. A direct correlation has been observed between the degree of lipid peroxidation and PLA_2-mediated hydrolysis of phospholipids [23]. On the other hand, inhibition of PLA_2 has been shown to significantly reduce lipid peroxidation [24]. Treatment with antioxidants attenuated viper venom-induced cellular damage by inhibiting the oxidative cascade and improving membrane stabilization [7, 25]. However, it is important to note that intravenously infused anti-venoms neutralize free and target-bound toxins but fail to counteract venom-induced inflammation and oxidative stress, as the antigen-antibody complex itself is pro-inflammatory [22]. Thus, a supplementary therapy is necessary to treat secondary and often overlooked complications of envenomation. The adjuvant antioxidant therapy must be provided as early as possible for more effective protection against ROS-mediated acute injury [26].

In conclusion, this study clearly demonstrated that systemic injection of EPV caused significant acute phase hepatotoxicity in mice. EVP-induced hepatic injury was accompanied with significant decrease in antioxidant enzymes activities, depletion of T-SH and increase in TBARS in liver, indicating the role of ROS in cellular damage. This acute phase oxidative stress points toward the importance of an early antioxidant therapy for the management of envenomation victims.

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Disclosure of conflict of interest

None.

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